Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 4638–4642, May 1992
Biochemistry

Polar zipper sequence in the high-affinity hemoglobin of *Ascaris suum*: Amino acid sequence and structural interpretation

(quaternary structure)

IVO DE BAERE*, LU LIU*, LUC MOENS*†, JOS VAN BEEUMEN‡, CONSTANT GIELENS§, JEAN RICHELLE¶, CLIVE TROTMAN‖, JOHN FINCH**, MARK GERSTEIN**, AND MAX PERUTZ**

*Department of Biochemistry, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium; ‡Laboratory of Microbiology, State University of Ghent, Ledeganckstraat 35, B-9000 Ghent, Belgium; §Laboratory of Biochemistry, Catholic University of Louvain, Dekenstraat 6, B-3000 Louvain, Belgium; ¶Department of Biochemistry, Free University of Brussels, Avenue P. Heger, B-1000 Brussels, Belgium; ‖University of Otago, Dunedin, New Zealand; and **Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, England

Contributed by Max Perutz, February 3, 1992

ABSTRACT **The extracellular hemoglobin of *Ascaris* has an extremely high oxygen affinity (P_{50} = 0.004 mmHg). It consists of eight identical subunits of molecular weight 40,600. Their sequence, determined by protein chemistry, shows two tandemly linked globin-like sequences and an 18-residue C-terminal extension. Two N-linked glycosylation sites contain equal ratios of mannose/glucosamine/fucose of 3:2:1. Electron micrographs suggest that the eight subunits form a polyhedron of point symmetry D_4, or 42. The C-terminal extension contains a repeat of the sequence Glu-Glu-His-Lys, which would form a pattern of alternate glutamate and histidine side chains on one side and of glutamate and lysine side chains on the other side of a β strand. We propose that this represents a polar zipper sequence and that the C-terminal extensions are joined in an eight-stranded β barrel at the center of the molecule, with histidine and glutamate side chains inside and lysine and glutamate side chains outside the barrel compensating each other's charges. The amino acid sequence of *Ascaris* hemoglobin fails to explain its high oxygen affinity.**

Hemoglobins are widely distributed among eukaryotes and have recently also been found in prokaryotes. Their amino acid sequences reveal that the globin fold has been conserved throughout evolution (1–3). Of the more than 500 amino acid sequences of globins in the latest National Biomedical Research Foundation Protein Identification Resource (NBRF/PIR) data base (release no. 29) (4), all except about 50 are from vertebrates and are either single-chain myoglobins or tetrameric hemoglobins. They are all very similar in sequence and structure, and their oxygen affinities are of the order of P_{50} = 0.5–1.0 mmHg (1 mmHg = 133 Pa). Invertebrate globins are more diverse in quaternary structure and oxygen-binding properties (for reviews, see refs. 1 and 5). They are mostly large extracellular aggregates classifiable by the number of chains and of globin domains in each chain (5). The 50 invertebrate globin sequences in the NBRF/PIR data base cover a wide range of phyla and types of quaternary structure. All retain the heme-linked proximal histidine (F8) and the phenylalanine (CD1) that wedges the heme into its pocket. Most of them also show the distal histidine (E7) and valine or isoleucine (E11), but more important, all retain a pattern of about 33 internal sites from which all polar residues except serine and threonine residues are excluded (6, 7).

Ascaris is a parasitic nematode that lives in the intestines of animals where oxygen is scarce. It contains a hemoglobin with an oxygen affinity two orders of magnitude higher than that of any vertebrate myoglobin or hemoglobin or any synthetic iron porphyrin made so far (P_{50} = 0.004 mmHg). We know of no way by which the oxygen affinity of ferrous heme could be raised to that level. As a first step toward the solution of this chemical mystery, we have determined the amino acid sequence of *Ascaris* hemoglobin.††

MATERIALS AND METHODS

The perienteric fluid hemoglobin of *Ascaris suum*, obtained in a local slaughterhouse, was prepared essentially according to ref. 8. The final preparation, which was passed over a Superose 6 column in 50 mM Tris·HCl, pH 7.5/100 mM NaCl, had an A_{412}/A_{280} = 1.3 and showed a single but broad spot on two-dimensional SDS/PAGE. The protein was split chemically or enzymatically. Chemical cleavage was done by CNBr, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine, or partial acid hydrolysis (9). Enzymatic cleavage was done on the denatured, unmodified maleylated protein, by either trypsin, chymotrypsin, or *Staphylococcus* V8 protease. Conformation-dependent cleavage was done with subtilisin (9). Specific cleavage after lysine was done with endoproteinase Lys-C (10). Analysis with carboxypeptidase P was done at pH 5.6 and 25°C with an enzyme-to-substrate ratio of 1:200. Peptide mixtures from chemical or enzymatic cleavage were separated by reversed-phase HPLC on C_4 (Vydac) or C_{18} (μBondapak; Waters) columns, and the peptides were eluted with acetonitrile in 0.1% trifluoroacetic acid at or near neutral conditions (50 mM triethylamine/acetic acid, pH 6.5). Several CNBr peptides were purified by preparative SDS/PAGE and electroelution according to refs. 11 and 12. Coomassie stain was displaced from the peptide with 1% SDS and extracted with four volumes of acetone. Excess SDS was extracted with acetone/acetic acid/triethylamine/water (17:1:1:1, vol/vol). Final contaminants were eliminated by reversed-phase HPLC. Endoproteinase Lys-C peptides, separated by two-dimensional SDS/PAGE were transferred by electroblotting (13). Amino acid compositions were determined by using a precolumn derivatization method (14). Tryptophan-containing peptides were identified by Ehrlich staining (10). Automated amino acid sequence determination was done by using a pulsed liquid-phase sequencer (Applied Biosystems models 477A and 473). Samples were run on Polybrene-coated glass fiber filters or on poly(vinylidene difluoride) membranes (13, 15, 16). Short peptides were sequenced manually by the 4,4-dimethylaminoazobenzene-4′-isothiocyanate/phenylisothiocyanate double-coupling method as described (17). Carbohydrates were analyzed by GLC on a CP Sil 5 fused silica WCOT

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: NBRF/PIR, National Biomedical Research Foundation/Protein Identification Resource.

††To whom reprint requests should be addressed.

‡The sequence reported in this paper has been deposited in the NBRF/PIR data base (accession no. A50001).

Table 1. Physicochemical characteristics of *Ascaris* globins

Parameter	Hemoglobin	Myoglobin
Native molecules		
$s_{20,w}$	11.6–11.8 (8, 23)	3.1 (24)
Molecular weight		
Equilibrium sedimentation	328,000 (8, 23)	37,000 (24)
Gel filtration	425,000 (25)	
pI	5.0 (26)	6.7 (26)
$\bar{v}$, g/ml	0.725 (27)	
f/f_o	1.51 (27)	
Heme per molecular weight	1/21,600 (23)	1/37,000 (24)
	1/40,000 (8)	
Maximum diameter by		
EM, Å	170 ± 15	
SAXS	170 ± 10 (28)	
Phenotypes, no.	1 (8)	2 (24)
Globin chains		
Molecular weight		
Equilibrium sedimentation	40,600 (27)	37,000 (24)
SDS/PAGE	43,000 ± 2000 (23)	
	38,500 ± 500 (25)	
Sequence	37,500	
Sequence including sugar	39,500	
% sugar, wt/wt	5.2	
pI	6.6 (25)	
Chains, no.	1 (25)	

The numbers in parentheses are the reference numbers from which the values were taken. Values without reference numbers were determined in this work.

(Chrompack, Delft, The Netherlands) column according to ref. 18. Protein concentration was based on amino acid analysis. The NBRF/PIR data base was used in combination with software written by Pearson and Lipman (19). Secondary structure prediction was done according to ref. 20. Molecular modeling, using the x-ray coordinates of sperm whale myoglobin, was done with the "Desktop Molecular Modeller" (21). The model of the β barrel was built with the program CHARMM (22).

RESULTS AND DISCUSSION

Ascaris hemoglobin is an octamer of $M_r \approx 328,000$ (Tables 1 and 2). Each subunit consists of a single polypeptide chain containing two glycosylated and covalently linked globin-like domains. It contains 311 amino acid residues with a calculated M_r of 37,461. Fig. 1 shows the reconstruction of the protein from representative peptides. Measurement of the carbohydrate content by GLC yielded a M_r of 2076, making the total M_r 39,537, compared with estimates of M_r 38,500 or 43,000 by dissociating polyacrylamide gel electrophoresis (23, 25) or M_r 40,600 by sedimentation equilibrium (23, 27). The high molecular weight of about 19,800 per globin domain is due partly to the carbohydrate and partly to an 18-residue extension of the second domain. The carbohydrates are attached to the side chains of the N-terminal asparagine and

Table 2. Ligand-binding properties of *Ascaris* globins at 20°C

Parameter	Hemoglobin O_2	Hemoglobin CO	Myoglobin O_2
P_{50}, mmHg	0.001–0.004	0.1 (28)	0.1 (29)
k'(on), $\mu M^{-1} \cdot sec^{-1}$	1.5	1.71 (29)	1.2 (29)
$t_{1/2}$, μsec	342	3.3 (29)	
k(off), sec^{-1}	0.0041	0.018 (29)	0.2 (29)
$t_{1/2}$, sec	173	139 (29)	

The numbers in parentheses are the reference numbers from which the values were taken. The values without reference numbers were taken from ref. 30.

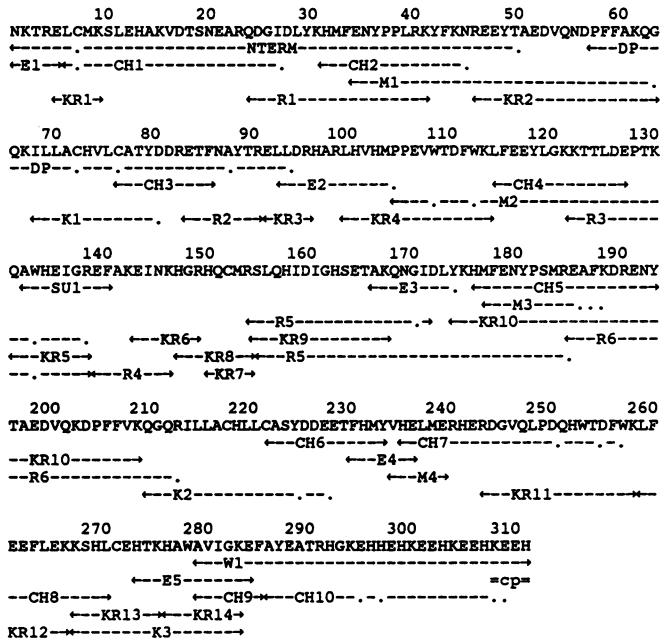


FIG. 1. Reconstruction of the *Ascaris* globin sequence from relevant peptides. Peptides were designated as follows: KR, tryptic; R, tryptic after maleylation; K, lysine-specific endoproteinase; CH, chymotryptic; E, *Staphylococcus* V8 protease; SU, subtilisin; M, CNBr; W, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; DP, partial acid hydrolysis. Peptides were numbered for each cleavage according to their position in the amino acid sequence of the chain starting from the N terminus. Dots indicate unidentified positions, and cp indicates carboxypeptidase B. Cleavage of the protein was as described in *Materials and Methods*.

asparagine CD6 (see Fig. 2 for residue notation) of the second domain. The ratio of mannose to *N*-acetylglucosamine to fucose is 3:2:1 (Table 3) (30).

Sixty-two percent of the residues in the two domains occupy identical positions (Fig. 2). They were aligned relative to template II of Bashford *et al.* (31), based on 226 vertebrate and invertebrate sequences, and compared to an alignment of 91 invertebrate globin domains. The alignment is confirmed (*i*) by the exclusion of polar residues from 30 out of the 33 invariant nonpolar sites listed in refs. 6 and 7; (*ii*) by the alignment of proline C2, which determines the folding of the BC corner; (*iii*) by the heme-linked histidine F8; (*iv*) by phenylalanine CD1, which is a heme contact invariably found in globin sequences; and (*v*) by the alignment of glycine residues B6 and E8, which are essential for the normal close contact between helices B and E. Alignment of the N-terminal 20 residues of the two domains to the classical globin sequence is ambiguous, mainly due to the lack of landmarks such as the conserved tryptophan A12. The best alignment, having no internal polar residues, creates an unusually long AB corner of 5 residues (Fig. 2). The influence of the glycosylated N-terminal residue and the structure of the first 20 residues defied prediction. There are no conflicting residues in the BC and CD region or in the D helix. The polar

Table 3. Carbohydrate content of *Ascaris* globin

Carbohydrate	Moles of carbohydrate per mole of protein or peptide: Native Hb	Peptide E1′	Peptide KR10
Fuc	2.32 (2)	1.01 (1)	1.45 (1)
Man	5.93 (6)	2.4 (3)	3.00 (3)
GlcNAc	2.52 (2 + 2*)	1.05 (1 + 1*)	1.06 (1 + 1*)

The numbers in parentheses indicate nearest whole numbers.
*The GlcNAc–Asn bond is not hydrolyzed.

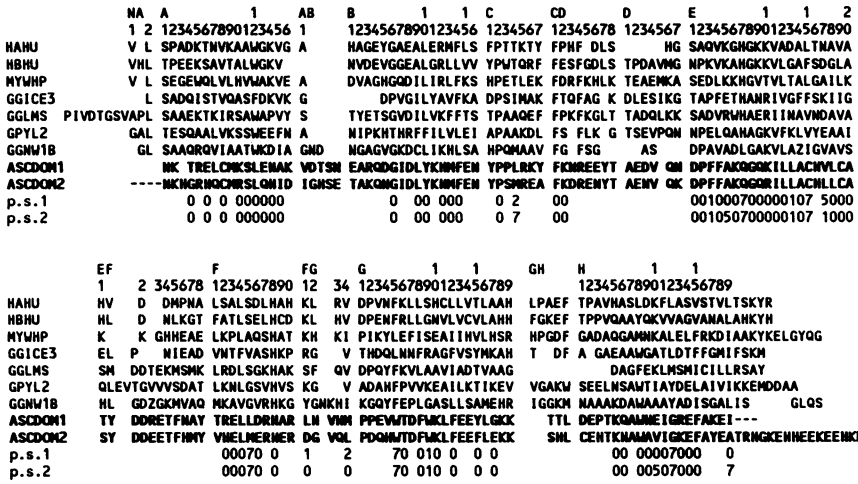


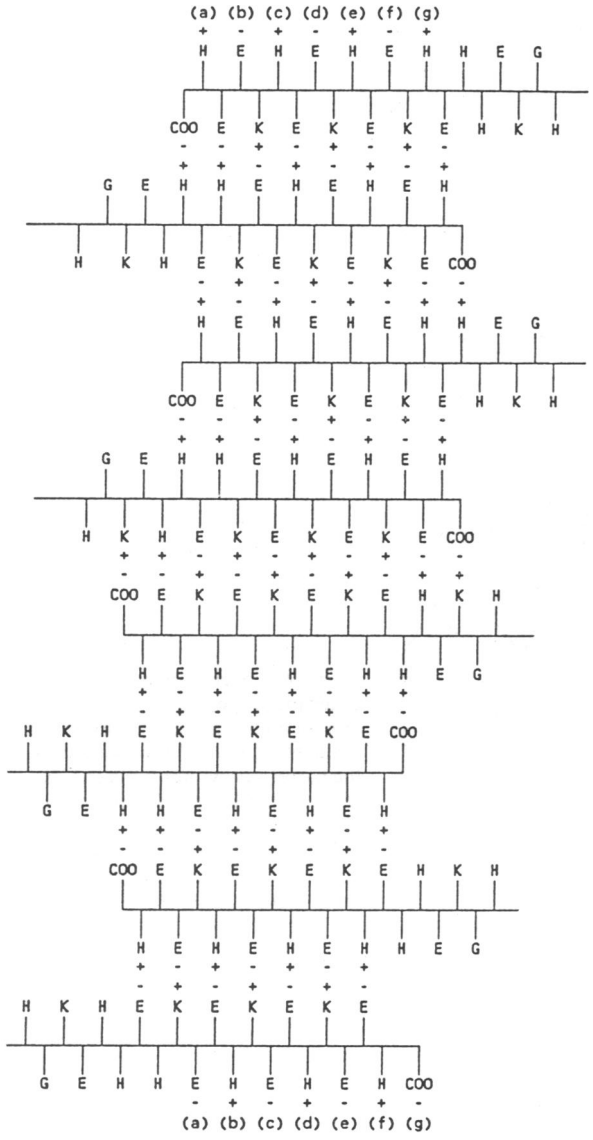
FIG. 2. Alignment of *Ascaris* hemoglobin domains with globins of known structure. The penalty scores for domains 1 (p.s. 1) and 2 (p.s. 2) are below the sequence. HAHU, human α chain; HBHU, human β chain; MYWHP, sperm whale (*Physeter catodon*) myoglobin; GGICE3, insect (*Chironomus thummi thummi*) globin III; GGLMS, lamprey (*Petromyzon marinus*) globin V; GPYL2, (*Lupinus luteus*) leghemoglobin II; GGNW1B, bloodworm (*Glycera dibranchiata*) globin major monomeric chain; ASCDOM1 and -2, domains 1 and 2 of common roundworm (*A. suum*) hemoglobin. The letters above the sequences indicate the helix notation.

residues that normally occupy positions E3, E13, and E17 are substituted by two phenylalanine residues and a leucine residue, indicating that helix E makes contact with a neighboring domain in the same polypeptide chain or with another subunit. The position of the usual distal histidine E7 is occupied by glutamine and that of the distal valine E11 by an isoleucine. These substitutions are rare in vertebrates (E7 is glutamine only in elephant myoglobin and in the α chain of opossum; E11 isoleucine occurs only in the α chain of opossum and in lamprey), but are more common in invertebrates. The internal cysteine residues E15 and E19 are too far apart to form a disulfide bridge. Tryptophan residues occupy positions G5 and G9 where they have not been found before. Normally G9 faces outward, which makes it likely that the indole ring of this tryptophan forms a domain or a subunit contact. If it were internal, space could be provided by its nearest neighbor, glycine H12, instead of the usual leucine, valine, phenylalanine, or tyrosine H12.

Most heme contacts are conserved, but some are unusual. At CD4 and F7 arginine residues in *Ascaris* replace phenylalanine residues in human hemoglobin. In both positions the phenyl ring is in a surface crevice, and the arginine side chains could arrange themselves with their guanidinium groups reaching into the solvent. The replacements of leucine FG3 in humans by glycine in *Ascaris* and of valine FG5 in humans by methionine or leucine in *Ascaris* may give the heme a tilt in a direction that, in human hemoglobin, has been observed to raise the oxygen affinity. Arginine CD4 could form a salt bridge with one heme propionate, and arginine E10 with the other propionate. The H helix of domain 1 is connected to the N terminus of domain 2. Due to the unusual A helix, it is hard to judge whether there is no linker sequence or whether the end of the H helix (from isoleucine H19) and a part of the A helix form the linker, but this does not resemble any of the linker sequences collated by Argos (32).

The C-terminal 18 residues have a regular repeat of $++--$. When this is plotted on a helical wheel, all the positively charged side chains come to lie on one face of the α-helix and the negatively charged ones lie on its opposite face, which would maximize electrostatic repulsion. On the other hand, if the sequence is plotted on a β strand, as in Fig. 3, then the charges on each side are positive and negative in turn, giving it maximal stability. Moreover, one side is lined by alternate glutamate and histidine residues and the other by alternate glutamate and lysine residues. To our knowledge, no such sequence has been reported. Its possible function is suggested by the quaternary structure of *Ascaris* hemoglobin. Darawshe and Daniel's (33, 34) electron micrographs indicate that the eight subunits of *Ascaris* hemoglobin, are arranged in two parallel layers; in each layer, four subunits lie at the corners of a square, consistent with the point group symmetry 42 (D4). They considered the two extreme arrangements with the squares in register ("eclipsed") or out of register ("staggered") and concluded that their micrographs were more consistent with the eclipsed arrangement. Our electron micrographs of *Ascaris* hemoglobin negatively stained with uranyl acetate, both pure and cross-linked with glutaraldehyde, are not consistent with an eclipsed model that would, at low resolution, have a center of symmetry and could not yield the frequent kidney-shaped and arrowhead-shaped images marked a and c, respectively, in Fig. 4. They are consistent with the staggered arrangement, with the two squares of subunits turned relative to each other by about 40°C as in Fig. 5, in views corresponding to the images a and c and to the image b, which is a view along the fourfold axis. Low-angle x-ray scattering of a concentrated solution of *Ascaris* hemoglobin yielded a radius of gyration of 54.4 ± 0.10 Å and a maximum dimension of 170 ± 10 Å (I. Pilz, E. Schwarz, I.D.B., and L.M., unpublished data). The average maximum dimensions measured on the electron micrographs are the same, with a scatter of about 15 Å.

The C-terminal sequence of *Ascaris* hemoglobin suggests that it acts as a cement between the eight subunits. It is suggestive that lysine and histidine residues are not mixed; since their side chains have different lengths, they would be misfits when juxtaposed. The eight β strands could be linked in a circle at the center of the octamer in one of two alternative antiparallel arrangements. In one of them, each β strand would be linked to its neighbor on one side by hydrogen bonds between the side chains of glutamate and histidine residues and to its neighbor on the other side by hydrogen bonds between glutamate and lysine residues (Fig. 3). The CO and NH groups of the main chains would remain free to form hydrogen bonds with water. Alternatively, these CO and NH groups would be hydrogen-bonded to each other in a β barrel as in Fig. 6 (22). Lysine side chains are on the outside (not shown) and histidine side chains, being shorter, are inside. Both are compensated by glutamate residues. The histidine and glutamate side chains could be fitted into the barrel in several ways without overcrowding. The tilt of the



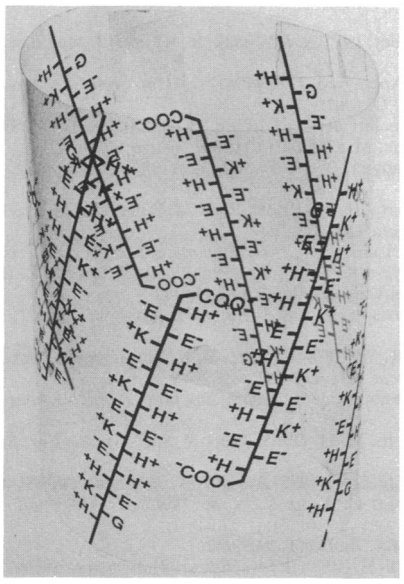


FIG. 3. (*Upper*) C-terminal sequence of *Ascaris* hemoglobin arranged on β strands. Note the alternation of cationic and anionic side chains on each side of the β strands, which suggests a zipper. (*Lower*) The sheet is rolled into a cylinder, with (a), (b), etc. at the top joined to (a), (b), etc. at the bottom.

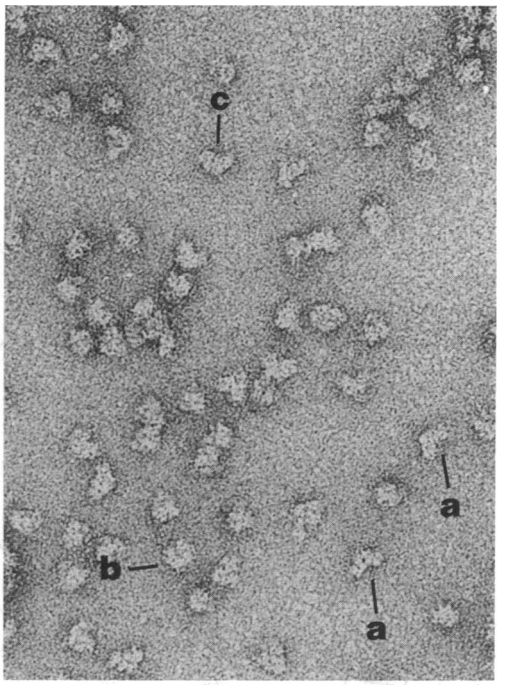


FIG. 4. Electron micrograph of *Ascaris* hemoglobin negatively stained with uranyl acetate. a, b, and c indicate kidney-, heart-, and arrowhead-shaped images, respectively.

chains was chosen to give good hydrogen bonding between neighboring β strands. Of the two alternative arrangements, the β barrel is the one likely to have the lower free energy. By analogy with the leucine zipper sequence that contains a leucine at every seventh residue along an α-helix, we propose to call the -Glu-Glu-His-Lys- repeat a polar zipper sequence.

The β-barrel model is consistent with the pH dependence of the sedimentation coefficient (23). This has a constant value of 11.7 S between pH 3.9 and about pH 8. Between pH 8 and 9, s drops to 11.2 S. Below pH 3.9 and above pH 11.5, the molecule dissociates into components of 4.6 S, corresponding to dimers, and 2.7 S, corresponding to monomers. The carboxylate of the glutamate side chain normally has a pK_a of 4.5, which would be lowered when it forms a salt bridge with a base. Dissociation due to discharge of the glutamate residues below pH 3.9 would therefore be consistent with our model. Free histidine and lysine side chains have pK_a values of 6.4 and 10.4, respectively, which would be raised by their being salt bridged to bases. A change of conformation at about pH 8 and dissociation into subunits above pH 11.5 is consistent with these pK_a values.

The very high oxygen affinity of *Ascaris* hemoglobin cannot be due to the replacement of the distal histidine by glutamine or of the distal valine by isoleucine, because in myoglobin this replacement *lowered* the oxygen affinity by a factor of 7 (35), and replacement of the distal valine by isoleucine in sperm whale myoglobin lowered the oxygen affinity more than 5-fold (36).

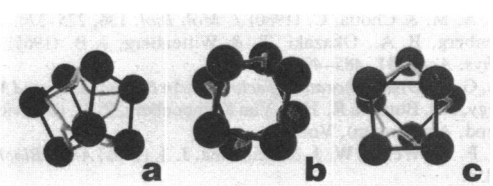


FIG. 5. Model of eight subunits as a polygon with D4, or 42, symmetry, viewed from three different angles, a, b, and c, on the electron micrograph.

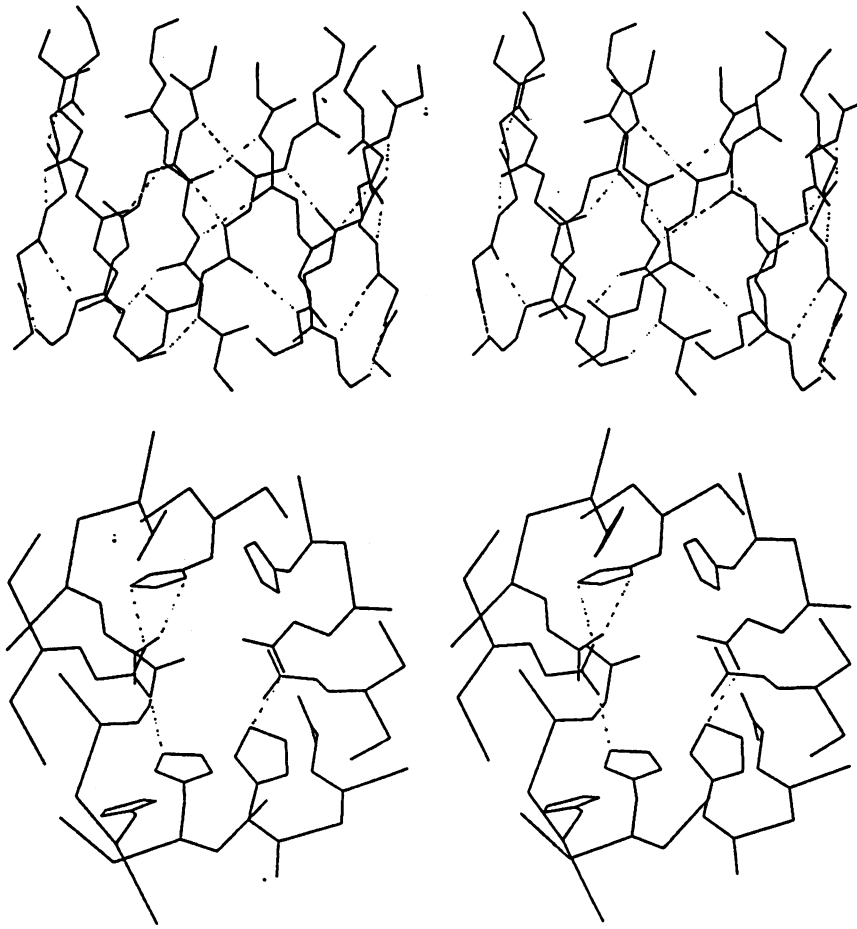


FIG. 6. Stereo diagrams of the C-terminal peptides assembled into an eight-stranded antiparallel β barrel. (*Upper*) View normal to the barrel axis without side chains. (*Lower*) View parallel to the barrel axis with glutamate and histidine side chains inside the barrel (shown) and lysine and glutamate side chains outside the barrel (not shown).

The tilt of the heme imposed by the replacement of valine FG5 by methionine or leucine, and of leucine FG3 by glycine, may raise the oxygen affinity, but not 100-fold. The mystery of *Ascaris* hemoglobin therefore remains unresolved. For its explanation, a crystal structure will be essential.

I.D.B. was supported by a grant from the Belgian Instituut ter aanmoediging van Wetenschappelijk Onderzoek in Nijverheid en Landbouw. L.M. and J.V.B. are supported by the Belgian Fonds voor Geneeskundig Wetenschapppelijk Onderzoek (Grant 3.0103.90). C.G. is supported by the Belgian Fonds voor Kollectief Fundamenteel Onderzoek. M.F.P. is supported by National Institutes of Health Grant HL 31461 and National Science Foundation Grant DMB 8916187. C.T. is supported by E. G. Johnstone and J. D. S. Roberts Trusts and by the Todd Foundation.

1. Goodman, M., Padweydon, J., Czelusniak, J., Suzuki, T., Gotoh, T., Moens, L., Shishikura, F., Walz, D. & Vinogradov, S. (1988) *J. Mol. Evol.* **27**, 236–249.
2. Runnegar, B. (1984) *J. Mol. Evol.* **21**, 33–41.
3. Riggs, A. F. (1990) *Am. Zool.* **31**, 535–545.
4. Barker, W. C., George, D. G., Hunt, L. T. & Garavelli, J. S. (1991) *Nucleic Acids Res.* **19**, 2231–2247.
5. Vinogradov, S. N. (1985) *Comp. Biochem. Physiol.* **82B**, 1–15.
6. Perutz, M. F., Kendrew, J. C. & Watson, H. C. (1965) *J. Mol. Biol.* **13**, 669–678.
7. Lesk, A. M. & Chotia, C. (1980) *J. Mol. Biol.* **136**, 225–270.
8. Wittenberg, B. A., Okazaki, T. & Wittenberg, J. B. (1965) *Biochim. Biophys. Acta* **111**, 485–495.
9. Allen, G. (1989) in *Laboratory Techniques in Biochemistry and Molecular Biology*, eds. Burdon, R. H. & Van Knippenberg, P. H. (Elsevier/North-Holland, Amsterdam), Vol. 9.
10. Jekel, P. A., Weijer, W. J. & Beintema, J. J. (1983) *Anal. Biochem.* **134**, 347–354.
11. Schägger, H. & von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379.
12. Hunkapillar, M. W., Lujan, E., Ostrander, F. & Hood, L. E. (1983) *Methods Enzymol.* **91**, 227–236.
13. Matsudaira, P. (1987) *J. Biol. Chem.* **262**, 10035–10038.
14. Cohen, S. A., Bidlingmeyer, B. A. & Tarvin, T. L. (1986) *Nature (London)* **320**, 769–770.
15. Hewick, R. M., Hunkapillar, M. W., Hood, L. E. & Dreyer, W. E. (1982) *J. Biol. Chem.* **256**, 7990–7997.
16. Hunkapillar, M. W., Hewick, R. M., Dreyer, W. J. & Hood, L. E. (1983) *Methods Enzymol.* **91**, 399–473.
17. Chang, J. Y., Brauer, D. & Wittmann-Liebold, B. (1978) *FEBS Lett.* **93**, 205–214.
18. Chaplin, M. F. (1982) *Anal. Biochem.* **123**, 336–341.
19. Pearson, W. R. & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2444–2448.
20. Rooman, M. J., Kocher, J.-P. A. & Wodak, S. J. (1991) *J. Mol. Biol.* **221**, 961–979.
21. Crabbe, M. J. C. & Appleyard, J. R. (1981) *Desktop Molecular Modeller* (Oxford Univ. Press, Oxford).
22. Brooks, B. R., Bruccoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S. & Karplus, M. (1983) *J. Comput. Chem.* **4**, 187–217.
23. Darawshe, S., Tsafadyah, Y. & Daniel, E. (1987) *Biochem. J.* **242**, 689–694.
24. Okazaki, T., Wittenberg, B. A., Briehl, R. W. & Wittenberg, J. B. (1967) *Biochim. Biophys. Acta* **140**, 258–265.
25. De Baere, I., Liu, L., Linnetop, L., Van Beeumen, J. & Moens, L. (1990) in *Invertebrate Dioxygen Carriers*, eds. Preaux, G. & Lontie, R. (Louvain Univ. Press, Louvain, Belgium), pp. 41–44.
26. Smith, M. H. & Morrison, M. (1963) *Biochim. Biophys. Acta* **71**, 364–370.
27. Okazaki, T., Briehl, R. W., Wittenberg, J. B. & Wittenberg, B. A. (1965) *Biochim. Biophys. Acta* **111**, 496–502.
28. Okazaki, T. & Wittenberg, J. B. (1965) *Biochim. Biophys. Acta* **111**, 503–511.
29. Gibson, Q. H. & Smith, M. H. (1965) *Proc. R. Soc. London Ser. B* **163**, 206–214.
30. Kornfeld, R. & Kornfeld, S. (1985) *Annu. Rev. Biochem.* **54**, 631–664.
31. Bashford, D., Chothia, C. & Lesk, A. M. (1987) *J. Mol. Biol.* **196**, 199–216.
32. Argos, P. (1990) *J. Mol. Biol.* **211**, 943–952.
33. Darawshe, S. & Daniel, E. (1990) in *Invertebrate Dioxygen Carriers*, eds. Preaux, G. & Lontie, R. (Louvain Univ. Press, Louvain, Belgium), pp. 251–254.
34. Darawshe, S. & Daniel, E. (1991) *Eur. J. Biochem.* **201**, 169–173.
35. Mathews, A. J., Rohlfs, R. J., Olson, J. S., Tame, J., Renaud, J.-P. & Nagai, K. (1989) *J. Biol. Chem.* **264**, 16573–16583.
36. Egeberg, K. D., Springer, B. A., Sligar, S. G., Carter, T. E., Rohlfs, R. J. & Olson, J. S. (1990) *J. Biol. Chem.* **265**, 11788–11795.